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(54) Bioelectrochemical assay electrode

(57) An electrode is, at least in part made from a material(X) having one-dimensional electrical conduction properties. The material X is conveniently an organic conductor, and preferably a derivative of 7, 7, 8, 8 tetracyano p-quinodimethane, especially in combination with one of the following ions or a salt thereof; Cu(di-pyridylamine), tetrathiafulvalene, ferricinium, triethylammonium or quinolinium. It may be a single crystal or packed into the cavity of a cavity electrode. The electrode may and comprise, at least at an external surface thereof the combination of an enzyme and a mediator compound which transfers electrons to the electrode when the enzyme is catalytically active. The additional material may be NAD^+/NADH couple, an oxidised/reduced flavin couple, or choline oxidase.

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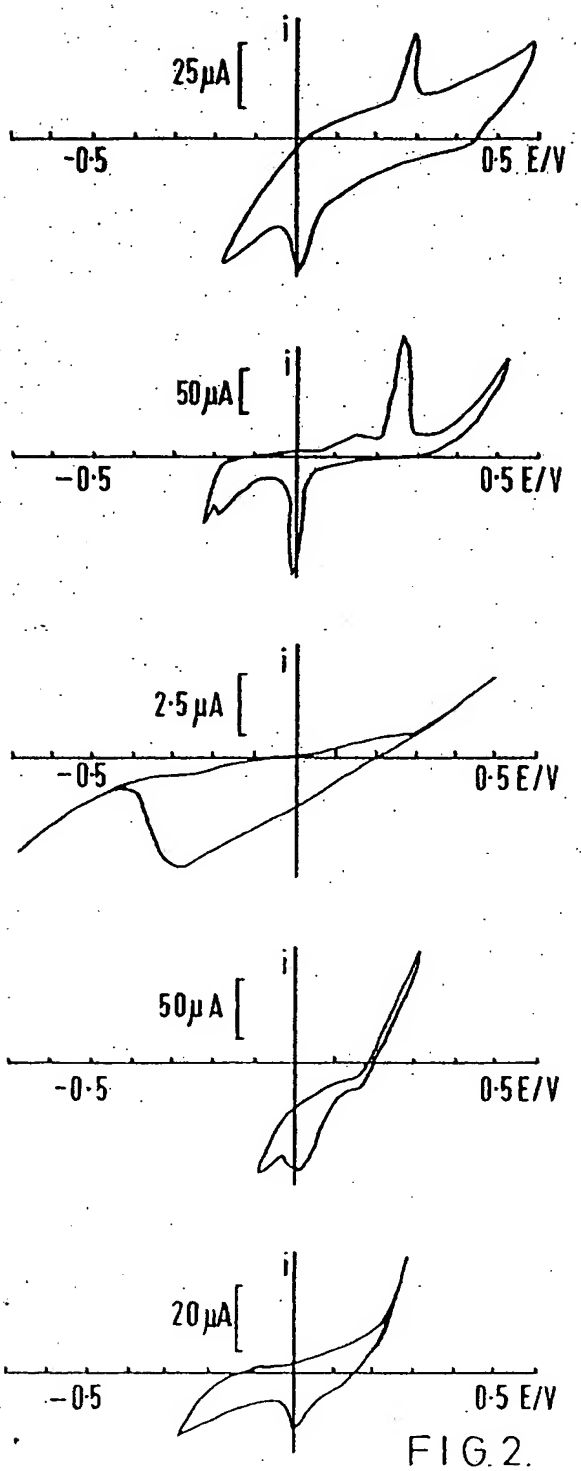
i
25μA
-0·5
0·5 E/V
A
i
50μA
-0·5
0·5 E/V
B
i
2·5μA
-0·5
0·5 E/V
C
i
50μA
-0·5
0·5 E/V
D
i
20μA
-0·5
0·5 E/V
E

FIG. 2.

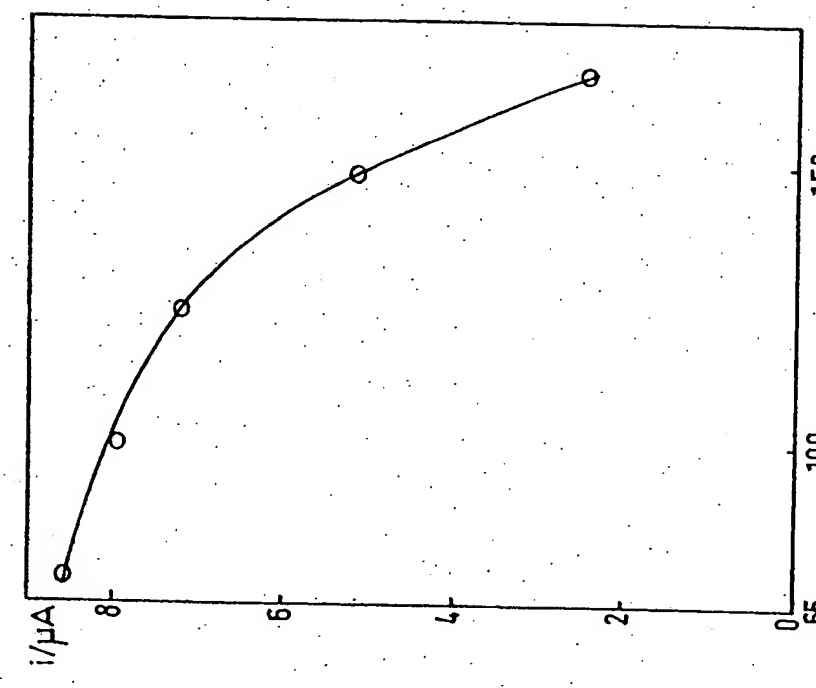
i/μA
8
6
4
2
0
65
100
150
TIME/hours

FIG.7.

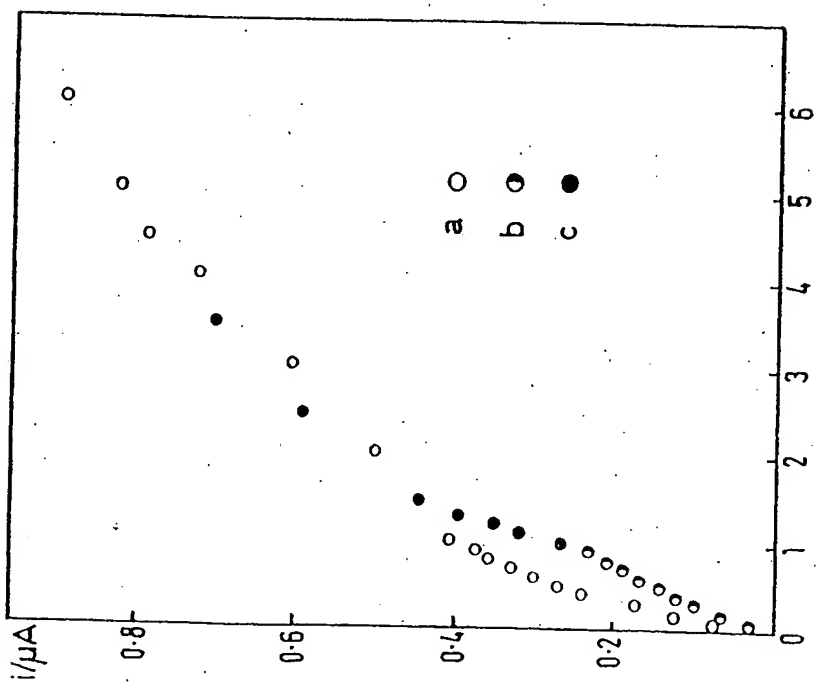
i/μA
0·8
0·6
0·4
0·2
0
1
2
3
4
5
6
glucose /mM
a
b
c

FIG.6.

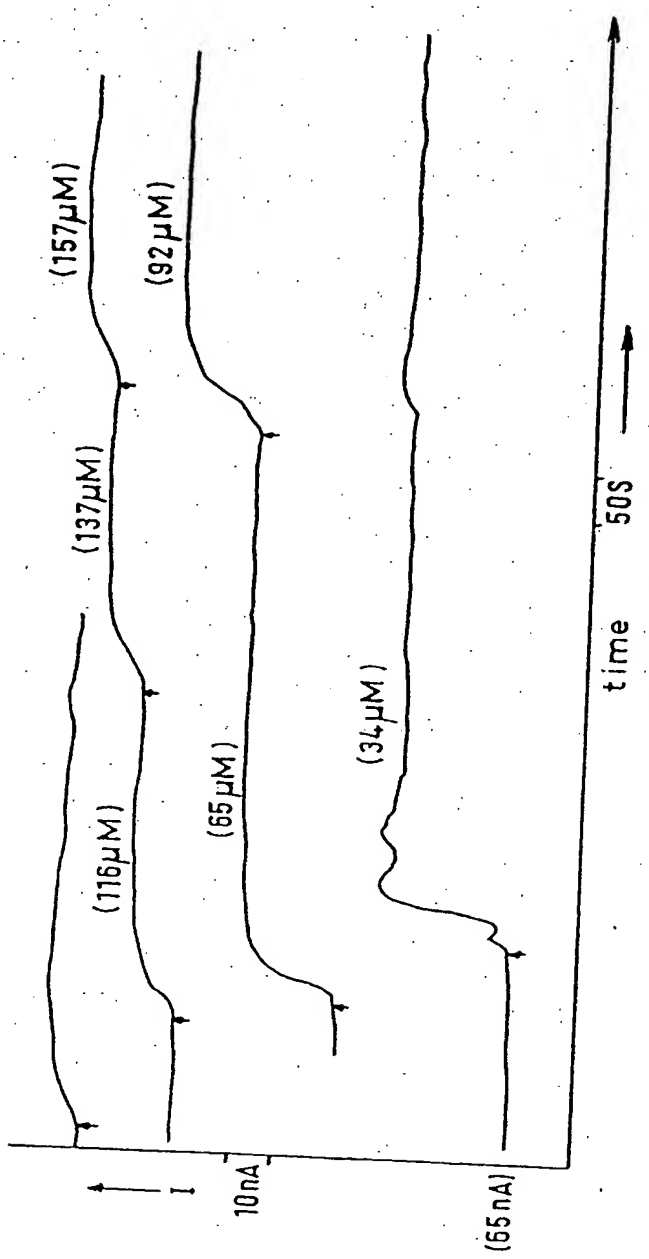

FIG. 10.

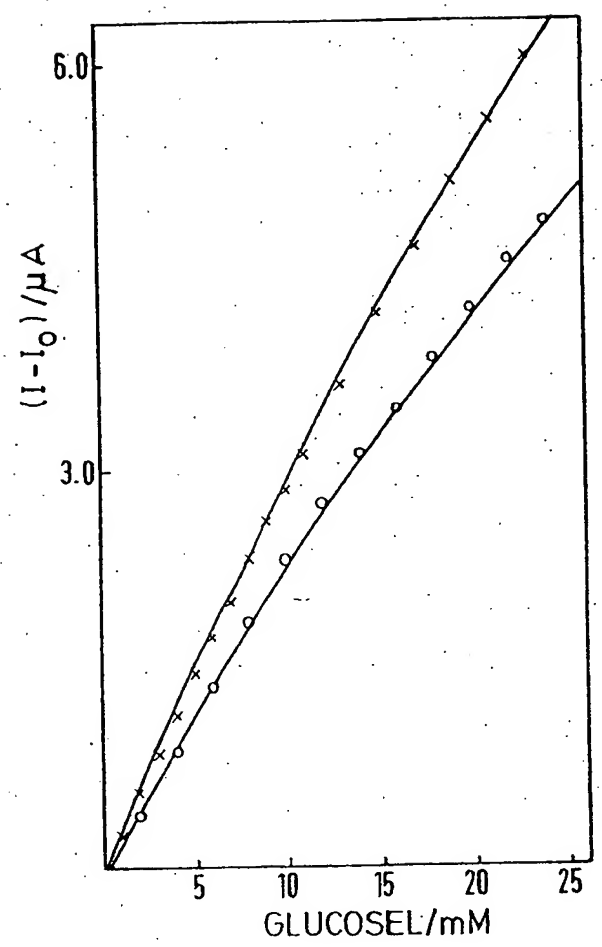

FIG.13.

x T=0

o T=65hrs Continuous operation

Conveniently the material (X) is an organic conductor.

Preferably the material (X) is a derivative of 7, 7, 8, 8 tetracyano p-quinodimethane.

One of the important requirements for an organic conductor was originally thought to be that the molecules of the solid had to have large planar molecules in which the valence electrons are found predominantly above and below the planar framework. One of the first organic molecules of this type to be synthesised was 7, 7, 8, 8-tetracyano-p-quinodimethane (TCNQ) which was found to a poor conductor of electricity.

More preferably the material (X) further comprises at least one of the following ions or a salt thereof; Cu(di-pyridylamine), tetrathiafulvalene, ferricinium, triethyl ammonium or quinolinium.

In a preferred embodiment of the invention, the material (X) comprises a tetrathiafluvaline (TTF) salt of 7, 7, 8, 8-tetracyano-p-quinodimethane

It has been determined that the salt TTFTCNQ is particularly stable, and is more stable than the other salts specifically exemplified herein. A particular utility of this compound is that it can be used in combination with a number of flavoprotein oxidases.

In one particular embodiment of the present invention the TTFTCNQ salt is used in combination with a flavoprotein selected from the following group; choline oxidase, zanthine oxidase, L-amino acid oxidase and D-amino acid oxidase.

In a further prefferred embodiment of the invention, the material (X) comprises an n-methyl phenazinum (NMP) salt of 7, 7, 8, 8-tetracyano p-quinodimethane.

NMPTCNQ was first prepared by Melby (Canadian Journal of Chemistry 1965, *43*, 1448) and was found to have a conductivity comparable to that of copper. Studies of the enzyme electrochemisty (Kulys et al.Anal Chim Acta 1982 *138* 19 and 1980 *117* 115) of this material have shown that it may enter into biochemical redox reactions, however no previous worker has shown that the material can be employed with an NADH -containing system.

We have determined that one particularly useful feature of the embodiments which employ NMPTCNQ is that the electrode potential may be swept outside of the region of electrode stability to dissolve the outer layers of the electrode in a controlled fashion, and thereby present a fresh surface to the electrolyte.

Accordingly, a further aspect of the invention resides in a method for the regeneration of an electrode for use in an electrochemical assay system, in which the potential of the electrode is swept outside of that range within which the outer layers of the electrode are stable to regenerate the electrode.

The above procedure is not possible with electrodes which have been modified with a covalent monolayer, or with a polymer layer containing redox groups.

In the solid form of the mixture, the TCNQ and for example TTF molecules, stack in separate, parallel columns and electrons are transferred from the TTF stack (donor) to the TCNQ stack (acceptor). Due to this electron transfer there can be a net motion of electrons along both stacks, hence the material is conductive.

This material was found to have the surprising property of anisotropic electrical conduction; that is, the material is highly conductive in one direction only, with the most favourable direction showing a five-hundred fold increase in conductivity over the least favourable direction.

We have demonstrated the general applicability of TCNQ containing assay systems when employed with oxidases and dehydrogenases, either when these are NAD-linked or are flavoproteins with other prosthetic groups.

Various configurations of electrodes can be envisaged within the scope of the present invention. For example the following general types of electrode; where the material (X) is packed as a paste into the cavity of a cavity electrode; where the material (X) is drop coated onto a glassy carbon electrode, or where the material (X) is present as a single crystal.

In the most preferrential embodiment of the invention the electrode further comprises an enzyme at least at an external surface thereof, whereby charge is transferred to the electrode when the enzyme is catalytically active. Preferably the enzyme is a flavoprotein, and is selected from the following group; Glucose Oxidase, Xanthine Oxidase, Choline Oxidase, L-amino acid Oxidase, D-amino acid Oxidase and Monoamine Oxidase.

All the materials studied, show reactivity as electrodes for the reoxidation of glucose oxidase. However in most cases the background currents were large and tended to drift. Thus one important feature in the choice of the TCNQ salt to be used as the electrode material is the background electrochemistry. For this reason TTF.TCNQ is the material of choice out of the five materials investigated.

A particularly useful and unexpected finding was that TTF.TCNQ could reoxidise choline oxidase, an enzyme for which no alternativee electron acceptor to O_2 was previously known. It is envisaged that an acetylcholine sensor could be configured by the use of choline oxidase in conjunction with acetylcholine esterase. Furthermore an acetylcholine esterase sensor can be envisaged which has a supply of acetylcholine provided at the electrode surface together with choline oxidase, and in which choline produced by the action of any added acetylcholine esterase is assayed as described herein.

NMP.TCNQ also works well with the other flavoproteins, in addition to glucose oxidase, for example, Xanthine Oxidase and Monoamine Oxidase.

The invention will be further described by way of example and with reference to the accompanying

EXAMPLE 3

Single crystal electrodes

For those materials which gave sufficiently large single crystals, electrodes were made up from these. Contact was made t6 one end of the needle-shaped crystals using a fine copper wire and a small quantity of silver-loaded epoxy resin. The contacted crystals were then carefully fitted into the ends of glass capillaries and insulated using ordinary epoxy resin so that about one half of the crystal was exposed to the solution. The whole electrode assembly was left to cure overnight and washed with DDW before use.

Electrochemistry

All measurements were made in phosphate buffer pH 7.4 containing 150mM NcCl. All potentials are reported relative to the saturated calomel electrode (SCE)

Figure 2 shows for comparison typical cyclic voltammograms for each of the electrode materials in background buffer. Cyclic voltammograms for the various salts recorded in phosphate buffer pH 7.4 at 25°C and 10 mV/s.

EXAMPLE 4

Use of Cu (dipyridylamine)TCNQ2

Cu(dipyridylamine)TCNQ was drop coated on glassy carbon (area 0.38 cm²).

In the background buffer solution the capacitative currents in the cyclic voltammogram were found to increase dramatically with successive cycles between −200 and +600mV. The background currents observed at a fixed potential were very slow to stabilize and particularly sensitive to the choice of potential.

Using a drop coated glassy carbon electrode in the presence of glucose oxidase a response to glucose was observed but this was far from ideal due to the high background currents.

EXAMPLE 5

Use of tetrathiafulvalene TCNQ.

TTF.TCNQ was drop coated on glassy carbon (area 0.38 cm²).

This material gave the best background electrochemistry with very low, stable background currents. It is the most promising of the compounds for detection of glucose using glucose oxidase of those so far investigated, including NMP.TCNQ. This work is described in detail in Example 9.

EXAMPLE 6

Use of ferricinium TCNQ

A ferricinium TCNQ single crystal was employed (area 2.0 mm²).

The packed cavity electrode method gave very poor results for this material. This appeared to be the result of the formation of free ferrocene on dissolution of the compound in tetrahydrofuran. For this reason all experiments with this compound were conducted with single crystal electrodes.

In background buffer the accessible potential range was approximately +500 to −700mV, the largest of any of the materials studied so far. In the presence of glucose oxidase and glucose in solution the electrode showed a response to added glucose. This response was not however well behaved under the conditions used.

EXAMPLE 7

Use of triethylammonium TCNQ

Triethylammonium TCNQ was drop coated on glassy carbon (area 0.38 cm²).

Of the compounds studied this was the least promising with very large cathodic background currents over nearly the whole "stable" potential range. This material was not investigated in any detail.

EXAMPLE 8

Use of quinolinium TCNQ.

Quinolinium TCNQ was packed into a cavity electrode (area 0.03 cm²).

The behaviour of this electrode in the form of a packed cavity electrode was almost identical to that of NMP.TCNQ both in the stable range for the material and in the results obtained in the presence of glucose oxidase and glucose.

Figure 3 shows typical data for detection of glucose using this material as a plot of the current (corrected for background) against concentration of glucose for a Quinolium TCNQ packed cavity electrode with Glucose Oxidase. Area 0.03 cm², E = 50 mV.

A: Where the electrode was covered with a dialysis membrane and a solution of 2.06 mg/ml Glucose Oxidase.

B: Where the electrode was dipped in 2.06 mg/ml Glucose oxidase for I hr and then washed before use without a membrane.

C: Where the same electrode as B was used, but after storage in buffer solution overnight.

9d) *Continuous operation*

Figure 12 shows the results of a further test into the stability of the electrode under conditions of continuous operation. Glucose Oxidase was the enzyme chosen in this case as it was the best characterised of the range of assay systems inventigated.

A 3.5mg/ml solution of glucose oxidase was entrapped on a TTFTCNQ packed cavity electrode using tissue paper and a membrane. The electrode was set up in a 20ml of degassed pH 7.4 phosphate buffer, background current was allowed to decay and additions of 1M glucose in phosphate buffer made. The electrode was then left at a constant potential of +50mV in a 30mM glucose solution for 65 hours. The glucose solution was then replaced by fresh buffer, the system was degassed and additions of 1M glucose were again made. The electrode was then left at the same potential for a further 100 hours of 40nM glucose solution at +50mV (Method of enzymatic analysis Vol II p.149 Verlay Chemie) and at room temperature. Each day the solution was degassed and the current recorded.

After 65 hours of operation the current/concentration profile showed a slight alteration in slope. Kinetic analysis of this data has suggested that this may be due to deterioration of the membrane. (Figure 13).

As a consequence of its low background the electrode described is sensitive to glucose concentration changes of less than 10 μM over a wide concentration range. It operates without a membrane or any additional mediator. The enzyme is irreversibly adsorbed onto the electrode and no special immobilisation techniques are required. The electrode shows excellent stability of response to glucose and upon prolonged storage (1 week) at room temperature in air-saturated buffer containing glucose. Finally when the electrode needs to be regenerated this is readily achieved by polishing the surface and then re-adsorbing glucose oxidase from solution.

EXAMPLE 10

Use of the electrode with other flavoproteins

In addition to elctrodes which employ Glucose Oxidase, the present invention extends to systems which combine TTFTCNQ with other enzymes. Four other flavoprotein/TTFTCNQ systems will be exemplified.

Packed cavity (4mm diameter) and drop coated glassy carbon electrodes were prepared substantially as described above. These electrodes were used in conjunction with a Pt gauze counter electrode, and a saturated calomel reference electrode in a three electrode system. The working electrodes were held at +50mV with respect to the saturated calomel reference electrode using a potentiostat.

Current was recorded as a function of time using a Bryans 29000 A4 chart recorder at 50s/cm. Packed cavity electrodes were used in a vessel of 25ml total volume; drop coated glassy carbon electrodes were used in a vessel of 2ml total volume. All experiments were carried out at room temperature.

Doubly distilled water was used throughout. Solutions were degassed before use by bubbling O_2 free N_2 through for 15 minutes The membranes used were dialysis tubing boiled in 1% W/W Na_2CO_3 for 10 minutes and stored in Tris (BDH)/EDTA solution.

EXAMPLE 10a)

Choline Oxidase (EC 1.1.3.17)

$$\text{Choline} + O_2 = \text{betaine aldehyde} + H_2O_2$$

Choline chloride and choline oxidase as used in this example were both obtained from Sigma. The choline oxidase used was 15u/mg. It shoudl be noted that there is no prior known electron acceptor, other than O_2 for choline oxidase.

A 1mg/ml solution of choline oxidase in pH 7.4 phosphate buffer was entrapped on a TTFTCNQ packed cavity electrode using dialysis membrane. The electrode was set up in 20 ml of degassed pH 7.4 phsophate buffer and background current was allowed to decay (to 10nA in 30 minutes). Choline chloride (0.1M in pH 7.4 phosphate buffer) was then added using a micro-litre syringe. A similar experiment was carried using an electrode which had been dipped in a 1mg/ml choline oxidase solution in an ice bath, for 1 hour in order to adsorb enzyme onto the electrode surface.

With the enzyme entrapped by a membrane the electrode responded to additions of choline (Figure 8). Without the membrane no response was obtained.

EXAMPLE 10b

Xanthine Oxidase (EC 1.2.3.2)

$$\text{Xanthine} + H_2O + O_2 = \text{urate} + H_2O_2$$

This enzyme exhibits low specificity and attacks a number of aldehydes, purines, pteridines, pyrimidines, ozapurines and other heterocyclic compounds. Ferricyanide, cytochrome c and several organic dyes can replace O_2 as an electron acceptor.

The materials used in this example were; xanthine (sigma grade III 98 - 100%), xanthine oxidase (Sigma grade III from buttermilk,suspension in 3.2 M $(NH_4)_2SO_4$ 10mM sodium phosphate buffer pH 7.8

blood sample from the finger, brings it into contact with the sensor, amplifies the signal and gives a digital readout.

CLAIMS

1. An electrode for use in an assay system, wherein the said electrode is at least in part made from a material (X) having one-dimensional electrical conduction properties, characterised in that, the material (X) is linked to the other components of the assay system via a $NAD^+/NADH$ couple.

2. An electrode for use in an assay system, wherein the said electrode is at least in part made from a material (X) having one-dimensional electrical conduction properties, characterised in that the material is other than the n-methyl phenazinum (NMP) salt of 7, 7, 8, 8-tetracyano p-quinodimethane or the n-methyl acridinium (NMA) salt of 7, 7, 8, 8-tetracyano p-quinodimethane.

3. An electrode for use in an assay system, wherein the said electrode is at least in part made from a material (X) having one-dimensional electrical conduction properties, characterised in that, the material (X) is linked to the other components of the assay system via an oxidised/reduced flavin couple.

4. An electrode as claimed in claim 1, 2 or 3, wherein the material (X) is an organic conductor.

5. An electrode as claimed in claim 4, wherein the material (X) is a derivative or salt of 7, 7, 8, 8 tetracyano p-quinodimethane.

6. An electrode as claimed in any of claims 1-5 wherein the material (X) further comprises at least one ion selected from the group comprising; Cu(di-pyridylamine), tetrathiafulvalene, ferricinium, triethylammonium or quinolinium.

7. An electrode as claimed in claim 6 wherein the material (X) comprises a tetrathiafluvaline salt of 7, 7, 8, 8-tetracyano p-quinodimethane.

8. An electrode as claimed in claim 1 or 3 wherein the material (X) comprises an N-methyl phenazinium salt of 7, 7, 8, 8 -tetracyano p-quinodimethane

9. An electrode as claimed in claim 1 or 3 wherein the material (X) comprises an N-methyl acridinium salt of 7, 7, 8, 8-tetracyano p-quinodimethane

10. An electrode as claimed in any of the previous claims, wherein the material(X) is packed as a paste into the cavity of a cavity electrode.

11. An electrode as claimed in claim 10, wherein;

a) a microcrystalline sample of the material(X) is mixed with polyvinyl chloride.

b) the resulting mixture is made up into a paste with tetrahydrofuran, and,

c) the said paste is packed into the cavity of the cavity electrode.

12. An electrode as claimed in claim 11 wherein the tetrahydrofuran is allowed to evaporate at room temperature and pressure.

13. An electrode as claimed in claim 11 or 12, wherein the ratio of material (X) to polyvinyl chloride is 9.1 : 1.4 by weight.

14. An electrode as claimed in any of claims 1-9, wherein the material (X) is drop coated onto a glassy carbon electrode.

15. An electrode as claimed in claim 14, wherein;

a) a microcrystalline sample of the material (X) is mixed with polyvinyl chloride,

b) the resulting mixture is made up into a liquid with tetrahydrofuran, and,

c) the said liquid is dropped onto the electrode, and the tetrahydrofuran is allowed to evaporate.

16. An electrode as claimed in claim 15, wherein a plurality of layers of the material (X) are applied to the electrode.

17. An electrode as claimed in any of claims 1-9, wherein the material(X) is present as a single crystal.

18. An electrode as claimed in claim 17 wherein:

a) a conductor is secured to a single crystal of the material (X) by silver-loaded epoxy resin, and,

b) the said crystal is fitted into one end of a glass capillary, with the said conductor internal to and co-axial with the said capillary such that substantially one half of the crystal is exposed.

19. An electrode as claimed in any of the preceeding claims further comprising an enzyme at least at an external surface thereof, whereby charge is transferred to the electrode when the enzyme is catalytically active.

20. An electrode as claimed in claim 19 wherein the enzyme is a flavoprotein.

21. An electrode as claimed in claim 19 or 20 wherein the enzyme is selected from the following group; Glucose Oxidase, L-amino acid Oxidase, D-amino acid Oxidase, Choline Oxidase, Xanthine Oxidase or Monoamine Oxidase.

22. An electrode as claimed in claim 19, 20 or 21, wherein a second enzyme is provided at or near the surface of the electrode to convert a substrate of the second enzyme to a substrate of the first-mentioned enzyme, and thereby provide a signal related to the concentration of the substrate of the second enzyme.

23. An electrode as claimed in claim 19, 20 or 21, wherein a substrate for a second enzyme is provided at or near the surface of the electrode, wherein the product of the second enzyme is a substrate of the first mentioned enzyme, whereby the electrode provides a signal related to the active concentration of the second enzyme.

24. An electrode for use in an assay system, wherein the said electrode is at least in part made from